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Use of a macromolecular crowding agent to dissect interactions and define functions in transcriptional activation by a DNA-tracking protein: Bacteriophage T4 gene *45* protein and late transcription

(T4 late genes/enhancers/replication–transcription coupling)

GLENN M. SANDERS, GEORGE A. KASSAVETIS, AND E. PETER GEIDUSCHEK

Department of Biology and Center for Molecular Genetics, University of California, San Diego, La Jolla, CA 92093-0634

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ABSTRACT **We have used a molecular crowding reagent to define functions in the transcriptional activation of bacteriophage T4 late genes. This activation normally requires the three T4 DNA polymerase accessory proteins encoded by T4 genes *44*, *62*, and *45* (the gp44/62 complex and gp45), an enhancer-like cis-acting site, an RNA polymerase-bound coactivator, and an unobstructed path along the DNA joining the promoter to the enhancer. We show that molecular crowding eliminates the requirement for the gp44/62 complex and for the enhancer, retains the requirement for gp45 and its coactivator, and generates activated promoter complexes with nearly unchanged DNase I footprints. These experiments identify gp45 as the direct activator of transcription, and the gp44/62 complex as the assembly factor for gp45. They suggest that the enhancer serves as the normal, but not invariably essential, entry site for the gp45 DNA-tracking protein.**

Transcription of the late genes of bacteriophage T4 is initiated at ≈40 extremely simple promoters, each one consisting of an 8-bp TATA box (TATAAATA in the nontranscribed strand) located ≈10 bp upstream of the transcriptional start site. Recognition of these promoters is conferred on the host RNA polymerase core (E) by the σ-family protein encoded by T4 gene *55* (gp55). Indeed, the E·gp55 RNA polymerase accurately initiates transcription *in vitro* at T4 late promoters in negatively supercoiled DNA (although this "basal" transcription is very inefficient in relaxed DNA and at high ionic strength). In contrast, expression of the T4 late genes in the virus-infected cell additionally requires the action of a second RNA polymerase-binding protein, T4 gp33, and concurrent T4 DNA replication. The coupling between DNA replication and late transcription can be broken in certain genetic backgrounds. However, even in such cases, late transcription is unconditionally dependent on the DNA replication protein gp45, one of the three proteins that confers processivity on the catalytic subunit of the T4 DNA polymerase holoenzyme (for reviews of T4 late transcription and T4 DNA replication, see refs. 1 and 2).

Analysis of T4 late gene transcriptional regulation *in vitro* has uncovered an interesting activation mechanism that requires the participation of the three DNA polymerase accessory proteins (gp45 and the gp44/62 complex) and their associated ATPase, the RNA polymerase-binding gp33, and an enhancer-like cis-acting site. The enhancer is an extremely simple structure, a nick or a single-strand gap that serves as the DNA loading site of the DNA polymerase accessory proteins and confers polarity on the transcriptional activation: the nick that constitutes the enhancer must be in the nontranscribed strand of its target transcription unit. Communication between a T4 late promoter and its enhancer requires a continuous and unobstructed path along the connecting DNA. The protein(s) that tracks along that path ultimately generates the open and enhanced T4 late promoter initiation complex, with gp45 situated at its upstream end, in the vicinity of the RNA polymerase-bound gp33, which is the coactivator of replication protein-dependent transcriptional activation (3–6).

A molecular model that accounts for the properties of these components has been proposed: the enhancer is the site at which the gp44/62 complex and gp45 bind to DNA and determines the orientation of that binding; ATP hydrolysis in the enhancer complex triggers the detachment of gp45, so that it is free to track bidirectionally along DNA; retention of the orientation that is imposed at the enhancer restricts productive contacts of gp45 to those molecules of gp33- and gp55-bearing RNA polymerase that face in the compatible direction (6). This model designates gp45 as the primary transcriptional activator, assigns an accessory assembly-factor role to the gp44/62 complex, and designates the enhancer as the DNA entry site for gp45.

The experiments that are described below analyze the consequences of perturbing the macromolecular interactions in this transcriptional activation system with a molecular crowding agent. We show that under conditions of macromolecular crowding, gp45 activates T4 late transcription in the absence of the gp44/62 complex. gp44- and gp62-independent activation of transcription dispenses with a requirement for the enhancer and for ATP hydrolysis and also eliminates the enhancer-imposed polarity of transcriptional activation.

MATERIALS AND METHODS

Polyethylene glycol (PEG) 8000, polyvinyl alcohol, dextran, and Ficoll were from Sigma. Solutions of these reagents were deionized on a mixed-bed resin (Bio-Rad AG501X8 D) before use. Other reagents were from standard sources. Nicked circular DNA was prepared as described (3). Linear DNA and relaxed circular DNA were prepared by digestion with *Sca* I restriction endonuclease and with calf thymus topoisomerase I, respectively. The relaxed state of DNA was verified by electrophoresis in agarose/ethidium bromide gels. Plasmids pDH310 and pDH82 have been described (4, 5). Their transcription units, yielding 420-nt RNA, have identical sequence.

RNA polymerase core was purified from uninfected *Escherichia coli* as described (5). gp55 was prepared as described (7), except that purified fractions were stored in 6 M guanidine hydrochloride in place of 8 M urea. gp33 was purified as described (8), with the addition of a final phenyl-Sepharose chromatography step to remove a minor contaminating nuclease activity. The gp44/62 complex was purified as described (5) from *E. coli* overexpressing T4 genes *44*, *45*, and *62*. In this purification method, gp44/62 is separated from

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gp45 at an initial anion-exchange step. gp45 came from two sources. The material for experiments shown in Figs. 1 and 3 was purified from *E. coli* bearing expression plasmid pOP45, which carries gene *45* under the control of a T7 RNA polymerase promoter. For the other experiments, gp45 was purified from the already-referred-to bacteria overexpressing both gp44/62 and gp45. Purification was according to Herendeen *et al.* (5), with the addition of a final step of Mono Q chromatography to remove a minor contaminating nuclease activity; this last step would also be expected to remove any residual gp44/62, although immunoblotting with rabbit antibody to gp44/62 prior to Mono Q chromatography found none [at a detection limit of 4 fmol of gp44 tetramer per reaction equivalent (3.6 pmol) of gp45].

For single-round transcription, 0.1 pmol of pDH82 DNA, 3.6 pmol of gp45 trimer, 1.7 pmol of gp44/62 complex, 1 pmol of RNA polymerase, 5 pmol of gp55, and 4 pmol of gp33 were combined on ice in 15 μl of transcription buffer [33 mM Tris·HOAc, pH 7.8/300 mM KOAc/10 mM $Mg(OAc)_2$/1 mM dithiothreitol containing bovine serum albumin (150 μg/ml)]. PEG 8000 (nominally 8000 number-average molecular weight) in transcription buffer was then added to the indicated final concentration in a 5-μl volume. After formation of the open promoter complex at 25°C for 45 min, single-round transcription was initiated by adding 5 μl of 5 mM GTP/5 mM ATP/0.5 mM [α-^{32}P]UTP (4000 cpm/pmol)/0.5 mM CTP/1 mM dithiothreitol containing rifampin (125 μg/ml) and RNase inhibitor (100 units/ml). RNA synthesis was ended 10 min later by adding 7 volumes of stop mix [0.4% (wt/vol) sodium dodecyl sulfate/20 mM Na_3EDTA/20 mM Tris·HCl, pH 8/250 mM NaCl, with yeast RNA (500 μg/ml)]. An aliquot of labeled DNA fragment was included in the stop mix as a recovery marker. Nucleic acids were analyzed and quantified essentially as described (3).

For DNase I footprinting, 0.1 pmol of nicked circular pDH310 DNA (whose single T4 late transcription unit is identical to the larger transcription unit of pDH82) was combined with 18 pmol of gp45 trimer, 4.2 pmol of gp44/62, 2.5 pmol of RNA polymerase, 12.5 pmol of gp55, and 10 pmol of gp33 in 37.5 μl of transcription buffer containing 10% (vol/vol) glycerol. PEG in transcription buffer was added to a final concentration of 10% (wt/vol), where appropriate, and the final volume was 50 μl. Open promoter complexes were formed during 45 min at 25°C. DNase I (3.75 ng in 10 μl of 0.5 mM $CaCl_2$) was then added for 30 sec, and digestion was stopped with 3 volumes of stop mix (transcription stop mix without carrier RNA). The footprint of the T4 late promoter was developed by primer extension using a 5′-end-labeled oligonucleotide complementary to the nontranscribed strand 138–156 nt downstream of the transcription start site. Reaction products were analyzed and quantified as described (9).

RESULTS

Pursuing experiments initially explored by D. R. Herendeen, we have examined the effects of the molecular crowding reagent PEG on transcription initiating at the bacteriophage T4 late promoters in plasmid pDH82. This DNA contains two T4 late transcription units in opposite orientation, yielding 420- and 314-nt transcripts (Fig. 1*A*). As noted in the Introduction, T4 late transcription *in vitro* is activated by three T4 DNA polymerase accessory proteins: gp45 and the gp44/62 complex. Transcriptional activation requires ATP (or dATP) hydrolysis and a nick, which serves as an enhancer-like site, in the DNA template; the DNA nick must be in the nontranscribed strand of the activated transcription unit. For pDH82, the nick (specifically introduced by the filamentous-phage gpII endonuclease) activated production of the 420-nt transcript in a single round of transcription, directly reflecting stimulated opening of the conjugate T4 late promoter as a consequence of transcriptional enhancement (Fig. 1*B*, compare lanes 1 and 3); all three DNA polymerase accessory proteins were required (compare lanes 3 and 2). The minor stimulation of the 314-nt transcript that can be seen in lane 3 was presumably due to adventitiously introduced nicks in the complementary strand of a small fraction of DNA templates (4). Addition of PEG, up to 10%, generated progressively more effective transcriptional activation in the presence of gp45 alone (lanes 8, 11, and 14). With 12.5% PEG, we encountered variably diminishing transcription (perhaps due to gross aggregation) (lanes 16–18 and data not shown).

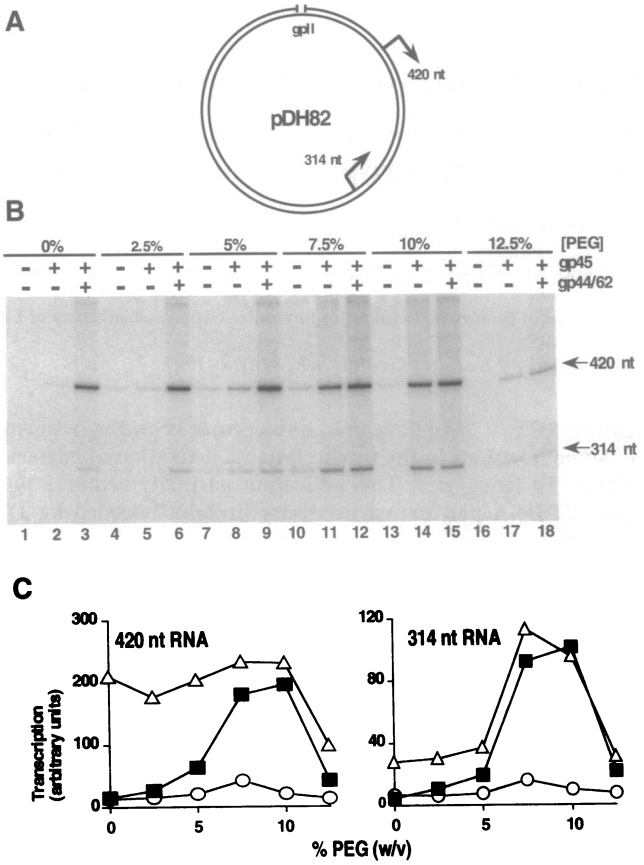

FIG. 1. Transcriptional activation by gp45 under conditions of macromolecular crowding. (*A*) pDH82 bears two T4 transcription units in opposite orientation and contains a unique gpII endonuclease site, generating a nick in the nontranscribed strand of the larger (420-nt) transcription unit. (*B*) Effect of PEG on enhancement of transcription by the DNA polymerase accessory proteins. Single-round transcription with E·gp55·gp33 was carried out in the presence of gp45, gp44/62, and increasing amounts of PEG as noted at the top. (*C*) Quantitative analysis of the 420- and 314-nt transcripts. Data from Fig. 1*B* were normalized to the recovery marker in each sample. ○, Unenhanced transcription; ■, transcription with gp45; △, transcription with gp44/62 complex and gp45.

Other macromolecular crowding reagents—polyvinyl alcohol, dextran, and Ficoll—also generated conditions under which gp45 activated transcription in the absence of gp44/62. Over the range of conditions explored by us, dextran and Ficoll yielded lower levels of activation by gp45 alone than did PEG, whereas polyvinyl alcohol yielded comparable levels of activation. On the other hand, glycerol, at concentrations up to 25% (vol/vol), did not generate gp44/62-independent activation of T4 late transcription, nor did very high concentrations of gp45 (up to 3 μM) in the absence of the molecular crowding reagent (data not shown). We found 10% (wt/vol) PEG 8000 to be optimal for transcriptional activation (Fig. 1*C*) and used this polymer concentration for the experiments that are described below.

Transcriptional activation by gp45 only in the presence of 10% PEG has been characteristically slightly less effective than transcriptional activation in the presence of excess gp44/62 complex in the same reaction medium (Fig. 1*C* and data not shown). The fact that the gp45 for this experiment was purified from *E. coli* overproducing only this protein eliminates the possibility that the macromolecular crowding reagents merely increase the efficiency of action of trace contaminating amounts of gp44/62. Moreover, the experiments described below demonstrate qualitatively distinctive properties for gp44/62-independent transcriptional activation.

The ATPase activity of the T4 DNA polymerase accessory proteins resides in the gp44/62 complex, which assembles with gp45 at primer–template junctions (refs. 10–14; for review, see ref. 2). As explained in the Introduction, the enhancer of T4 late transcription is thought to serve as the assembly site of this complex (3), and an ATP hydrolysis-dependent process is thought to provide DNA entry to gp45, which must track along DNA in order ultimately to become a component of the enhanced transcriptional initiation complex at the T4 late promoter (4, 6). Transcriptional activation that is independent of the gp44/62 complex might also be independent of the enhancer (note the progressively increasing yield of the 314-nt transcript in Fig. 1*B*, lanes 8, 11, and 14) and might not require ATP hydrolysis. The next two experiments demonstrate that this is the case.

The results of a comparison of transcriptional activation in circular nicked, relaxed but covalently closed, and linearized pDH82 DNA are shown in Fig. 2. Transcriptional activation in the absence of PEG required nicked DNA and was asymmetric—that is, effective for generating the 420-nt transcript only (Fig. 2, compare lane 1 with lanes 5 and 9). In 10% PEG, gp45 alone stimulated transcription of all three kinds of DNA efficiently, symmetrically increasing production of both the 314-nt and the 420-nt transcript in parallel (compare lanes 4, 8, and 12 with lane 1); the gp44/62 complex alone was without substantial effect on transcription (compare lanes 3, 7, and 11 with lanes 2, 6, and 10, respectively). Additional transcripts were generated in linear DNA (compare lanes 9 and 5) and this activity was also stimulated by gp45. We suggest that variant late promoters in pDH82 are the source of this transcription (3), that transcription of circular DNA initiating at these promoters yields long transcripts running in an upper part of the gel that is not shown in Fig. 2, but that these promoters yield readily detectable, shorter run-off transcripts on *Sca* I-linearized DNA. That transcriptional activation by gp45 is independent of ATP hydrolysis was confirmed by the experiment shown in Fig. 3: the activation occurred in a reaction medium containing the nonhydrolyzable ATP analogues adenosine 5′-[γ-thio]triphosphate and

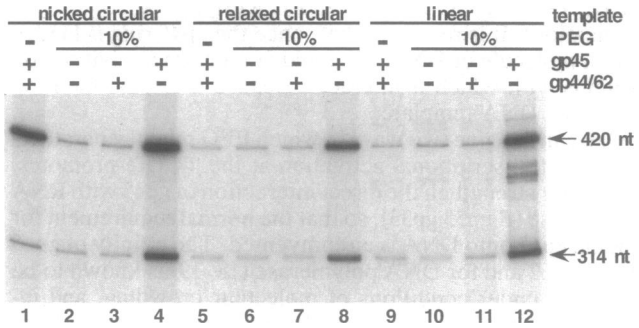

FIG. 2. PEG allows activation of transcription on relaxed circular and linear DNA. Single-round transcription assays were carried out as described in Methods on nicked circular (lanes 1–4), relaxed circular (lanes 5–8), and linear (lanes 9–12) DNA in the presence and the absence of 10% PEG.

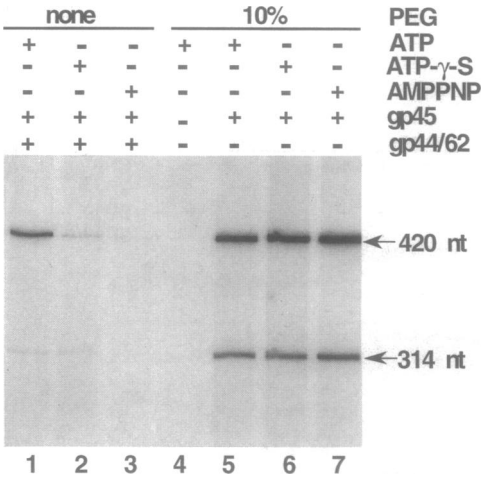

FIG. 3. Transcriptional activation by gp45 alone does not require ATP hydrolysis. Lanes 1–3, single-round transcription in the absence of PEG and in the presence of gp45 and gp44/62; lanes 4–7, transcription with 10% PEG and gp45. Lanes 1 and 4, transcription with 1 mM ATP; lanes 2 and 5, with 200 μM adenosine 5′-[γ-thio]triphosphate (ATP-γ-S) replacing ATP; lanes 3 and 6, with 200 μM adenosine 5′-[β,γ-imido]triphosphate (AMPPNP) replacing ATP. (ATP-γ-S and AMPPNP serve as substrates for RNA polymerase but do not support hydrolysis of the β-γ phosphoanhydride bond.)

adenosine 5′-[β,γ-imido]triphosphate with 10% PEG present (lanes 6 and 7), but not in the absence of the macromolecular crowding reagent (lanes 2 and 3).

Enhancerless and gp44/62 complex-independent transcriptional activation in a macromolecular crowding medium appears to involve a strikingly different mode of delivery of gp45 to the T4 late promoter. Nevertheless, as the next two experiments show, the interactions that are ultimately generated at the promoter under these very different reaction conditions are strikingly similar. In particular, transcriptional enhancement in the presence of PEG continued to depend on the RNA polymerase-bound coactivator gp33 (Fig. 4). In medium with PEG, as in conventional reaction media (5), gp33 depressed unenhanced transcription (compare lanes 4 and 2). gp45 was ineffectual as an activator (compare lanes 3 and 2) unless gp33 was also present (compare lanes 5 and 3). The slightly (1.6- to 2-fold) stimulated transcription generated by gp45 in the absence of gp33 (lane 3) was consistently observed in four other experiments. This modest effect may indicate that gp45-mediated transcriptional enhancement is greatly potentiated by gp33 but not exclusively mediated through gp33.

The physical structures of enhanced promoter complexes generated with and without the participation of the gp44/62

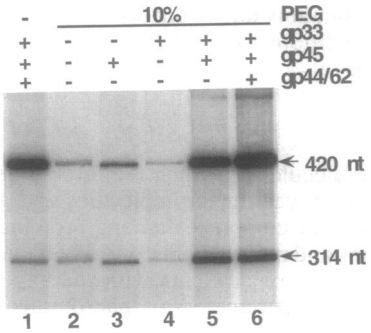

FIG. 4. Activation of transcription in the presence of PEG requires the coactivator gp33. Products of single-round transcription with the components indicated above each lane are shown.

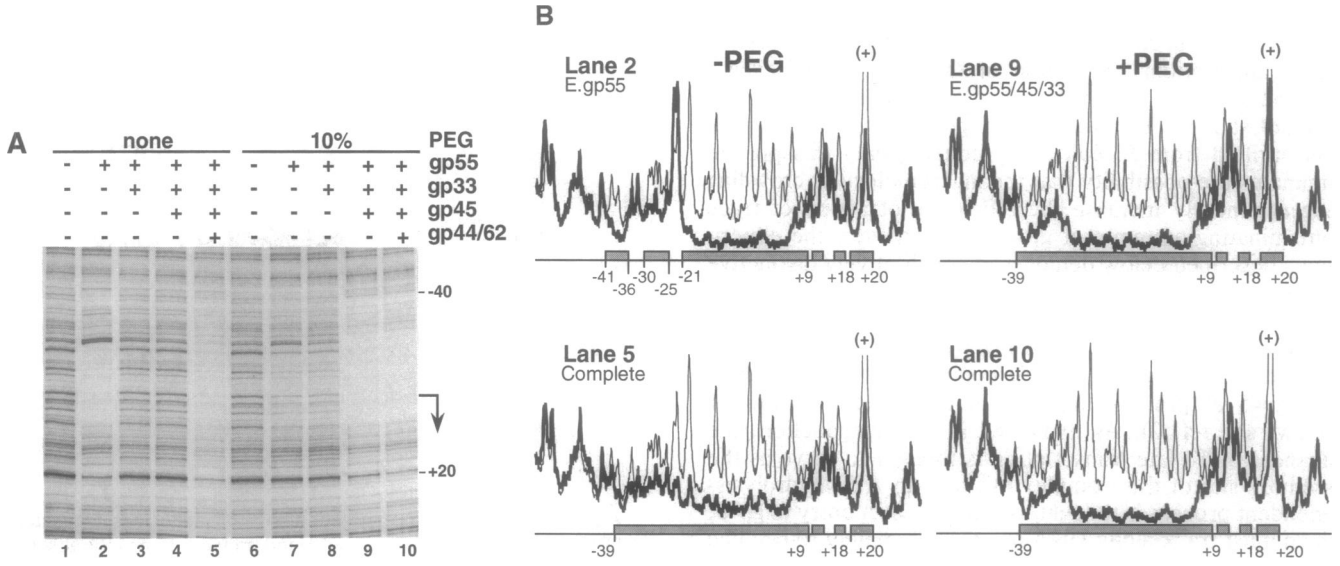

FIG. 5. DNase I footprints of open promoter complexes assembled in the presence and absence of PEG. (*A*) Open promoter complexes were assembled on the T4 late promoter in pDH310 that yields 420 nt RNA, using nicked DNA, RNA polymerase core, and the indicated components. The presence of 10% PEG, gp55, gp33, gp45, and gp44/62 with RNA polymerase core is specified at the top. Arrow at right indicates the start point (+1) and the direction of transcription. (*B*) Densitometric scans of footprints from lanes 2, 5, 9, and 10. Tracings of the respective no-protein control lanes (thin lines) are superimposed after having been aligned and normalized at the flanks of the footprint. Regions of protection are summarized below each trace.

complex were also strikingly similar when analyzed by DNase I footprinting. Footprints of the nontranscribed strand of the promoter yielding the 420-nt transcript in 10% PEG with and without gp44/62 were nearly identical (Fig. 5*A*, compare lanes 9 and 10, and Fig. 5*B*). The enhanced transcription complex formed in the absence of PEG likewise generated the same footprint, albeit less completely (lanes 5 in Fig. 5 *A* and *B*). As noted previously (6), unenhanced and enhanced transcription complexes differed markedly only upstream of bp −21 (compare lane 2 with lanes 5, 9, and 10 in Fig. 5 *A* and *B*): contiguous protection from bp +9 to −39 in the enhanced complex, but hyphenated protection between bp −21 and −41, with enhanced DNase cleavage at bp −22 and −23 in the unenhanced complex. The same, but less complete, unenhanced footprint was also generated in the presence of PEG (Fig. 5*A*, lane 7); the lower promoter occupancy of this sample (compare lane 7 with lane 2) was consistently observed but has not been analyzed further. This difference between the footprints of unenhanced and enhanced complexes upstream of bp −21 has been ascribed to gp45, and the presence of gp45 in that vicinity has been directly demonstrated by photocrosslinking (6). We note that the footprints upstream of bp −31 in the unenhanced complex and downstream of +9 in unenhanced and enhanced complexes on pDH310 (Fig. 5) differ in detail from what has been recently reported for a closely related promoter in plasmid pRT510 (6), presumably due to differences of DNA sequence and reaction conditions (R. L. Tinker, unpublished work in our laboratory).

DISCUSSION

Conditions of molecular crowding reinforce otherwise tenuous interactions between macromolecules and generally favor the formation of complexes among proteins, as well as between proteins and nucleic acids (15). We have undertaken the above-described experiments with the expectation that manipulations of the parameters of macromolecular interaction in our *in vitro* system might yield new perspectives on transcriptional activation by the T4 DNA replication proteins. Our expectations have been realized, in that we have found the gp44/62 complex to be dispensable for activation of T4 late transcription under conditions of macromolecular crowding (e.g., in 10% PEG; Fig. 1). Without gp44/62, there is no need for ATP hydrolysis or for the enhancer (Figs. 3 and 2, respectively); without an enhancer there is no way to generate polarity of transcriptional activation (Fig. 2); transcriptional activation by gp45 alone continues to require gp33 (Fig. 4). All of these findings are entirely consistent with the model of transcriptional activation already discussed in the Introduction, in which the normal role assigned to the gp44/62 complex is that of loading gp45 onto DNA at the enhancer.

A concrete and compelling model of gp45 loading currently dominates thinking about this process. T4 gp45 is a homologue of prokaryotic and eukaryotic DNA replication proteins that generate highly processive nucleotide addition by DNA polymerases: the β dimer of the *E. coli* DNA polymerase III holoenzyme and PCNA, the proliferating-cell nuclear antigen of eukaryotic DNA replication. Each of these proteins is loaded onto DNA at a nick by its conjugate gp44/62 homologue in an ATP hydrolysis-requiring reaction (reviewed in ref. 16). The β dimer is a six-domain ring with a central hole large enough to admit double-stranded DNA (17). Similar structures have been postulated for the gp45 trimer and for PCNA (16) and have recently been confirmed for PCNA (T. S. R. Krishna and J. Kuriyan, personal communication). It is reasoned that, once the gp45 ring is loaded onto a molecule of DNA, it should confer processivity upon proteins with which it interacts because it retains them on that same DNA template.

One can imagine two ways in which PEG might generate its effect on transcriptional activation at the T4 late promoter. (*i*) It might strengthen the direct interaction of gp45 with RNA polymerase (E·gp55·gp33), so that the normal requirement for loading gp45 onto DNA is circumvented. The affinity of gp45 for gp44/62 and for DNA polymerase (18, 19) is known to be increased under conditions of molecular crowding, and increased interaction between gp45 and E·gp55 has also been noted (J. Winkelman, unpublished work in our laboratory). (*ii*) Molecular crowding might allow gp45 to load onto DNA directly without the intervention of assembly factors. Although efficient assembly of toroidal β dimer onto circular DNA requires the DNA polymerase III γ complex and ATP

hydrolysis (20–22), it may also take place at very low efficiency in the absence of the γ complex (23). Another DNA-encircling complex, the double-ring RuvB dodecamer, can also assemble around DNA in the absence of its targeting cofactor, RuvA (24). It is conceivable that macromolecular crowding might facilitate gp45 loading onto DNA either through the formation of higher-order structures or by stabilizing the interaction of gp45 with DNA-bound RNA polymerase. The first of the above alternatives implies that the structures of the gp45-only and gp44/62-assembled enhanced promoter complexes might be different; the second alternative implies identical or nearly identical structures. In fact, the corresponding DNase I footprints are strikingly similar in that they share the upstream extension to bp −39 (Fig. 5*B*); this is space that is occupied by gp45 in the gp44/62 assembly-assisted promoter complex (6).

It has been shown that gp45 increases the processivity of T4 DNA polymerase much more effectively under conditions of macromolecular crowding than in the absence of the crowding agent. PEG, at concentrations similar to those used in this work, allows the requirement for gp44/62 in strand displacement during synthesis of DNA to be bypassed. Moreover, as already mentioned, conditions of macromolecular crowding promote the interaction between gp45 and T4 DNA polymerase (19). These parallels have led some of us to speculate that the mechanism by which gp45 enhances transcription and the mechanism by which gp45 generates processive DNA synthesis are identical: both occur because gp45 retains weakly interacting proteins on the DNA template (6, 25). Although gp45 does not provide an auxiliary upstream DNA-binding site for the E·gp55·gp33 RNA polymerase in the manner of the *E. coli* cAMP receptor protein, CRP, (26, 27), it is capable of generating a comparable effect on transcriptional initiation by effectively confining the RNA polymerase–promoter interaction to a one-dimensional space in place of a three-dimensional space.

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